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Characterizations of the active ingredients of methanol extract of weaver ant and its analgesic activity in mice

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Abstract: Pain according to WHO has been one of the greatest issues to plague man, in the bid to handle this issue of pain, man has sought to look for other means to reduce pain to its bare minimum. This study is aimed at investigating the analgesic activity of the methanolic extract of the African weaver ant using acetic acid induced writhing, hot plate method, and formalin induced pain models in Swiss mice. In the acetic acid test, the methanolic extract of Oecophylla longinoda (OL) was administered orally at 200 and 400 mg/kg body weight while aspirin was administered at 100 mg/kg and tween 80 served as standards. In the hot plate and formalin models, the extract was administered orally at two doses of 200 and 400 mg/kg while pentazocine at 10 mg/kg and tween 80 at 10 mg/kg served as standards. The methanolic extract of OL exhibited significant analgesic activity in all the models, with none less than the standard significant difference ($p < 0.05$) by increasing the reaction time of the mice after treatment in comparison to the control. The 400 mg/kg extract in the acetic acid induced writhing response has a percentage inhibition of 52.7%, which shows how well the extract inhibits pain in mice. The methanolic extract significantly reduced pain response in mice, with a p-value of 0.03, 0.02, and 0.001 in all the test models, respectively. OL increased the pain threshold over time and significantly reduced the writhing response that mice experience from acetic acid. Furthermore, pretreatment with OL significantly and dose-dependently decreased the early and late phases of formalin-induced pain in mice. Thus, these findings suggest that the methanolic extract of OL acts on central and peripheral pain pathways.

Introduction

Pain is described by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with an actual or potential tissue damage [1]. One of the most prevalent signs of sickness or injury is pain; it acts as a natural alarm and defense system and is frequently helpful in establishing a correct diagnosis. Analgesia has been the subject of numerous studies, yet pain management is still one of medicine's trickiest and most pervasive issues. For hundreds, if not thousands of years, opiates have been the primary method of pain relief. However, using them comes with a cost that includes physical dependence, respiratory problems and immune system depression [2]. As a result, research to discover new alternatives to treat pain is crucial and to develop potent substances that will address the biggest threat to human health, according to the World Health Organization (WHO).

The pain threshold is a value that determines whether mechanical, thermal, chemical or electrical stimulation causes the release of pain mediators from the affected tissue. Individual pain thresholds and pain tolerance must be distinguished to account for the fact that each person experiences pain differently for a given noxious stimulus [3]. The threshold of pain is the point at which pain begins to be felt. It is a wholly irrational phenomenon. The threshold intensity is the level at which a stimulus (such as heat or pressure) starts to cause pain. The pain threshold, like other sensory thresholds, is generally constant, but the pain tolerance level, which is defined as the amount of pain a subject is willing to tolerate, varies greatly. In clinical practice, patients typically wait until they have reached their pain tolerance level before seeking medical help. By employing a visual - analogue pain scale, an individual can often be measured at that level of pain [4]. Numerous human disorders exhibit pain as a symptom and various analgesics are frequently used to treat pain. Traditional pain therapies rely on opioid analgesics, cyclooxygenase inhibitors, nonsteroidal anti-inflammatory drugs and analgesic adjuvants, which include several classes of compounds that were introduced to pain therapy from the arsenal of drugs used to treat other medical conditions but have been found, in some cases, in the clinical setting, to be effective in the pain control [5]. Analgesics are medications that reduce or remove the pain that many pathologic illnesses cause. It is challenging to enumerate all the circumstances in which analgesics are required and where addiction is not likely, such as when treating muscle aches and headaches. Opioids, which primarily affect the central nervous system and nonopioids, which mostly affect the peripheral nervous system, are two categories of analgesics [6].

The test models used here are the acetic acid induced writhing, which is a chemical method used to induce pain of peripheral origin by injection of irritant principles like acetic acid, phenylquinone in mice. The test compound's analgesic effectiveness can be derived from a decrease in frequency of writhing's [7]. Initial description of the abdominal writhing in mice included an arched back, extended hind limbs, and contracted abdominal muscles. Another method used here is the hot plate test, which utilizes latency measurements to assess acute, cutaneous pain sensitivity; however, the behavioral reactions that were assessed in this test are structured supraspinally [8]. Finally, the formalin induced pain models is a tonic model of persistent pain brought on by tissue damage caused by formalin. It is a naturally occurring pain-related reaction that can be seen in an uncontrolled, free-moving animal. Behaviors can be rated over a protracted period such that the precise start and duration of analgesics can be assessed. Given that it includes inflammatory, neurogenic, and central pathways of nociception, it is a helpful model, especially for the screening of novel drug compounds [9].

In order to choose the best treatment for a patient, it is essential to identify the most likely pain mechanism during clinical assessments [10]. As a result, a list of clinical signs generated from expert consensus has been produced as the criteria on which clinicians may make their judgements for acceptable categorization [11]. Smart et al. [11] identifies subjective and objective clinical signs for each of the three types of pain: central, peripheral neuropathic and nociceptive. A primary lesion or central nervous system malfunction is what causes or initiates central pain [11, 12]. Peripheral neuropathic pain encompasses a variety of pathophysiological pathways linked to altered nerve functioning and responsiveness and is triggered or induced by a primary injury or dysfunction in the peripheral nervous system. Mechanisms include mechanical, thermal, chemical and impulse creation that is aberrant and hyperexcitability [11, 13]. When painful chemical (inflammatory), mechanical, or ischemic stimuli are present, the peripheral receptive terminals of primary afferent neurons become activated, resulting in nociceptive pain [11, 14]. Herbal medicines have been utilized for healing for millennia. Several of these plants were utilized for

their analgesic properties without any negative side effects. Natural products are chemical molecules created by life and they are separated into bacteria, animals and plants [15]. Recent studies have revealed the presence of bioactive chemical constituents to include alkaloids, terpenoids, flavonoids and steroids while antimicrobial activity have also been reported for the methanol extract of *Oecophylla longinoda*, OL [16, 17]. Scientists have spent a lot of time and effort over the years investigating the novel and active compounds found in plants; while much has been accomplished in this area, much more has to be done. Several animal species also have active compounds that could be used, especially those in the class, Insecta. Thus, this study aimed at determining the analgesic activity of the methanolic extract of the African weaver ant using acetic acid induced writhing, hot plate method and formalin induced pain models in Swiss mice.

Materials and methods

Collection and identification of the ant: The ants were gathered from an almond tree (*Terminalia catappa*) in the Nigerian state of Edo, near the Ikpoba settlement of Ikpoba Okha local government on the 18th of February 2022 (**Image 1**). A zoologist at University of Benin's, Department of Animal and Environmental Biology in Benin City, Nigeria, identified the ants as Oecophylla longinoda (Weaver ants, OL) and a specimen has been kept at the Department as a reference.

Image 1: Oecophylla longinoda (Weaver ant, Nigeria)

Preparations and extraction: Fifteen ant nests were gathered and submerged in distilled water. After sorting the leaves, the ants were filtered out and the leaves were ground in a mortar. 600 ml of methanol were used to thoroughly extract 64.9 g of the crushed ants over the course of an 8-hour period in a Soxhlet extractor. To create a semi-solid extract. The crude extract was concentrated using rotary evaporator (Model RE, 200, USA) at 50 °C.

Fractionations of the crude extract: The crude methanolic extract was fractionated into hexane and ethyl acetate fractions using separating funnel. The recovered fractions from the methanol extract of OL were evaporated using rotary evaporator, the two portions were condensed and characterized using gas chromatography mass spectrometry (GC-MS).

Experimental animals: Mature Swiss mice (Mus musculus) weighing 25 - 35 g of either sex obtained from the animal house of Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, were used. Mice were housed in plastic cages with sawdust as beddings and kept in a room with controlled temperature (25 ^{0}C) under a 12 : 12 hour light/dark cycle and a free access to food and water supplied *ad libitum*. Prior to use, mice were left to acclimatize for two weeks. All procedures described were reviewed and approved by the ethics committee (see below).

Animal study: Animals were randomly assigned into four groups of five mice each group for the three different experimental models. First group served as negative control receiving Tween 80. Second group served as positive control and given standard drugs, 10 mg/kg pentazocine and 100 mg/kg aspirin, respectively. The extract of OL was administered at 200 mg/kg to third group and 400 mg/kg to the fourth group. Treatments given orally and intraperitoneally. The selected doses chosen upon pilot studies.

Preliminary bioactive chemical screening: The screening for secondary metabolites was carried out to detect the presence or absence of different bioactive constituents from the methanolic extract of OL [17, 18].

GC-MS: Chemical constituents of both fractions were analyzed using Gas Chromatography (Agilent 7890B) equipped with a HP-5 ms ultra-inert column and coupled to Mass Spectrometer (Agilent 5977A). The sample was dissolved in methanol solvent prior to analysis.

Acetic acid induced writhing: The method used was early described by Koster [19]. Mice were divided into four groups of five each group. Tween 80 was used as the control and administered to group one. Groups two and three received 200 and 400 mg/kg of the extract, respectively, while 100 mg/kg of aspirin, an analgesic was given to the last group. All administrations were done orally. One hour after pre-treating the mouse, 0.6% of acetic acid was given intraperitoneally at dose of 10 ml/kg of body weight. Mice were isolated in individual cages and observed for 30 minutes, during which the number of writhes were counted. The mean number of writhes was calculated and the percentage inhibition of writhes was determined as following:

$$\% \text{ of inhibition } = 100 - \binom{Xt}{Xc} x\ 100$$

Where Xt is writhes of the test and Xc is the writhes of the control.

Hot-plate method: The method used is as described [20] and then modified [21]. Following an initial screening, the mice were divided into four groups of five each. The animals were first gently dropped on the hot plate at 54.8 ± 0.5 ^{0}C [21] to obtain their initial reaction time (time in seconds for the mice to either try to jump out or lick its hind paw). Thereafter, mice were then treated as such: group 1: 10 ml/kg of tween 80 (orally), group 2: 10 mg/kg of pentazocine (intraperitoneally), groups 3 and 4: 200 and 400 mg/kg of the extract (orally), respectively. Mice were again placed on the hot plate at 60, 90, 120, 150 and 180 min following the extract intake and the mean values were recorded.

Formalin induced pain: This method was described by Shibata [22]. Swiss mice were divided into four different groups of five animals each. The first group was pretreated orally with 0.1 ml of tween 80, the second group was given the reference drug, pentazocine, intraperitoneally at 10 mg/kg, the third and fourth groups received the extract orally at 200 and 400 mg/kg, respectively. After one hour, 0.02 ml of 01.0% formalin was injected subcutaneously into the right hind paw of the mice in all the groups. Thereafter, the time in seconds spent in licking the injected paw was taken as an indicator of the pain response. These responses were measured first for five minutes after formalin was injected indicative of the first phase (neurogenic pain) and then for another 15 - 30 minutes as second phase (inflammatory pain).

Evaluation of acute toxicity: The acute toxicity determination was conducted by applying the novel approach to practical acute toxicity testing according to Lorke et al. [23].

*Ethical considerations***:** The permission to conduct the current study has been obtained from Faculty of Pharmacy, Animal Use and Ethics Committee of University of Benin with permit reference number EC/FP/022/23.

Statistical analysis: All the data were presented as mean ± standard error mean (SEM). The results have been analyzed using a Statistical Package for the Social Sciences (SPSS) version 19. The statistical significance difference between the parametric groups was determined by using Student's t-test. Differences among the groups calculated by one-way analysis of variance (one way-ANOVA) followed by Dunnett's post hoc test. A p value of less than 0.05 was considered significant.

Results

Preliminary bioactive chemical screening: As shown in **Table 1**, the presence of saponins, alkaloids, phenolics, flavonoids, glycoside, terpenoids and eugenols were all confirmed through the qualitative color changes of test reagents which will give a clue to the possible mechanisms of the analgesic effects of the methanolic extract of OL.

Table 1: Bioactive constituents of OL

Bioactive chemicals	Methanol
Alkaloids	+
Glycosides	+
Saponins	+
Phenolics	+
Terpenoids	+
Eugenols	+
Steroids	-
Flavonoids	+
Tannins	-
+ = present	**- = absent**

GC-MS: **Figure 1** indicates certain components detected from the isolated oil fraction (hexane fraction) of the methanolic extract of OL were found as octanoic acid (RT, area: 13.476, 25.48%), cis-vaccenic acid (RT, area: 32.046, 16.81%), 9-octadecenoic acid (Z), methyl ester (RT, area: 31.177, 16.61%), oleic acid (RT, area: 33.125, 13.17%) and hexadecanoic acid, methyl ester (RT, area: 29.554, 6.10%).

Figure 1: GC-MS spectrum of hexane (oil) fraction of OL

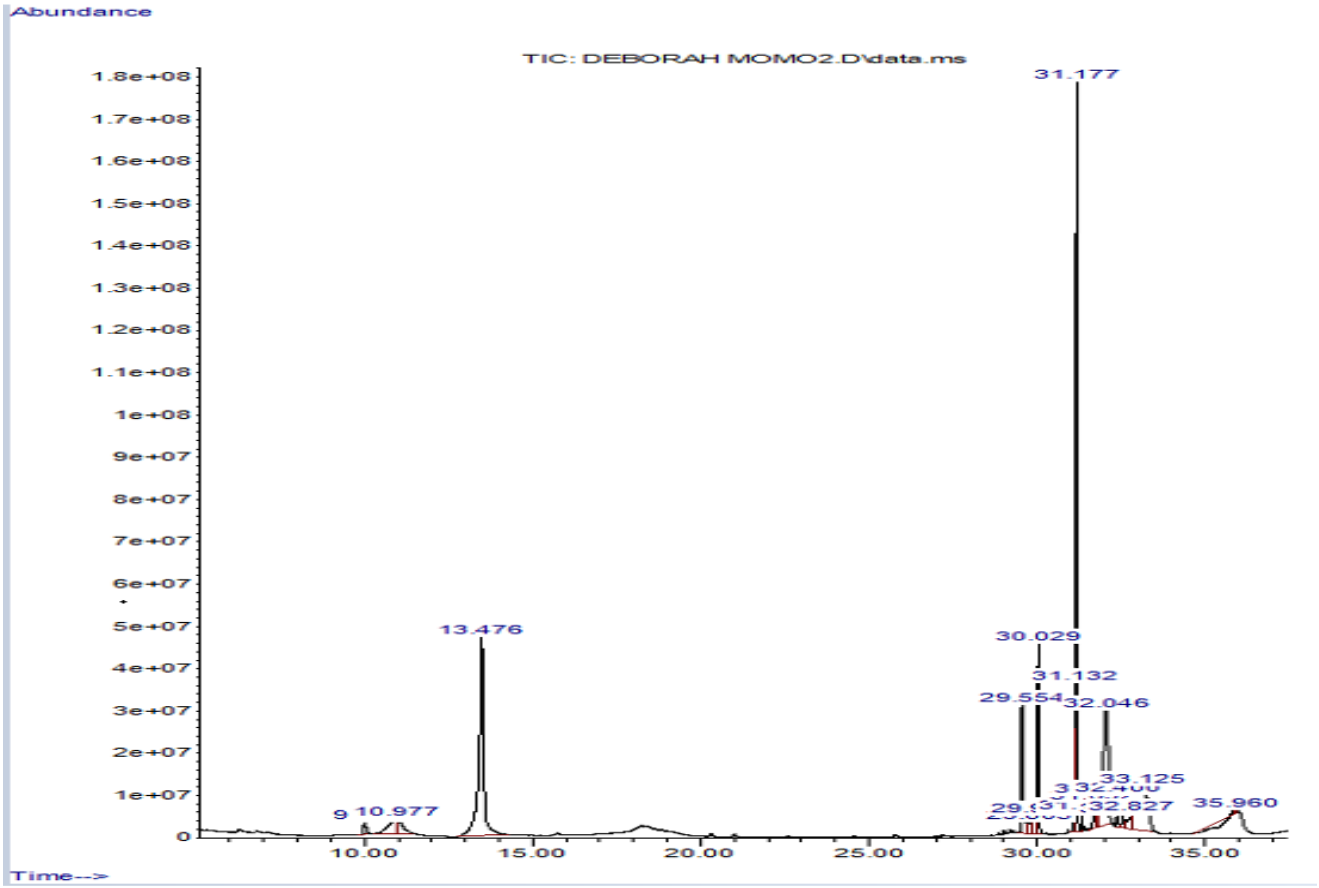

Figure 2 shows some major components detected from the ethyl acetate fraction of OL were (-) -carvone (RT, area: 13.547, 21.12%), oleic acid (RT, area: 33.960, 10.84%), oleic acid (RT, area: 32.273, 7.16%), oleic acid (RT, area: 35.320, 5.25%), tricosane (RT, area: 32.394, 5.74%), cis-11-Hexadecenal (RT, area: 33.000, 4.94%), oleic acid (RT, area: 34.126, 4.81%), hexadecanoic acid, methyl ester (RT, area: 29.548, 4.65%), D-carvone (RT, area: 13.403, 4.31%). In **Figure 3**, oleic acid, a naturally occurring monounsaturated fatty acid, interacts with the vanilloid (capsaicin)-binding pocket to suppress TRPV1 activity as well as pain and itch responses in mice.

Acetic acid-induced writhing test: In this model, central and peripheral analgesics is chemically induced nociception. As shown in Table 2, methanol

extract of the OL inhibited the writhing response induced by an intraperitoneal injection of acetic acid in mice at a dose of 10 mg/kg with inhibition percentage of 29.3% and 52.7%, respectively. The extracts significantly decreased writhing counts in mice above the standard drug of choice. The positive drug, aspirin at 100 mg/kg, even though it is significantly inhibited the writhing response, did not serve as a good inhibitor like the methanol extract. Therefore, the results showed that all the doses of the extract tested significantly inhibited the acetic acid induced writhing in mice as there was a reduction in the number of writhes when compared to the control and the standard drug. When compared with the reference drug aspirin, the number of writhes counted for the 400 mg/kg group was significantly lower with percentage inhibition of 52.7% compared to 21.2% for aspirin.

Hot-plate test: Table 3 displays the findings of the hot plate method used to determine the analgesic effect of the methanol extract of OL. The findings revealed that over the 180 minutes observation period, there was a discernible difference in the mice treated with the extract in response to the heat shock. Within the same treatment groups, there was increase in reaction time over baseline values (0 min) at any point in time. As a result, the considerable increase in the reaction time to thermal discomfort was noticeable in the extract treated animal groups. Oral administration of the extract at a dose of 200 mg/kg produced significant inhibition of the hot-plate response from the first hour, which reached a peak 1 hour 30 mins after administration (8.90 ± 1.46, one-way ANOVA followed by Dunnett's post hoc test, pb 0.01). 200 and 400 ml/kg of the extract markedly prolonged latencies of the hot-plate paw-licking response from the first to the third hour after oral administration, reaching a peak of 9.19 ± 1.76. Overall, the extract dose-dependently increased latencies of the hot-plate paw-licking response. The antinociceptive effect of the extract treated group was fast and long-lasting, demonstrated by the fact that the extract group produced marked anti-nociception as early as the first hour after oral administration and the effects remained significant up to the third hour after administration. The progressive increase over the course of the observation period shows that like the standard, pentazocine, the extract elicited considerable analgesic effect within a short time of treatment. Pentazocine-treated mice displayed analgesic action at about the same rate as the 200 mg of extract, however, the 400 mg displayed a higher analgesic effect. This goes to show that the latency of the hot-plate paw-licking response in the extract-treated group (400 ml/kg) was greater than both the pentazocine and the 200 mg/kg extract group and it reached statistical significance (one-way ANOVA followed by Dunnett's post hoc test, ($p < 0.02$).

Figure 2: GC-MS spectrum of ethyl acetate fraction of OL

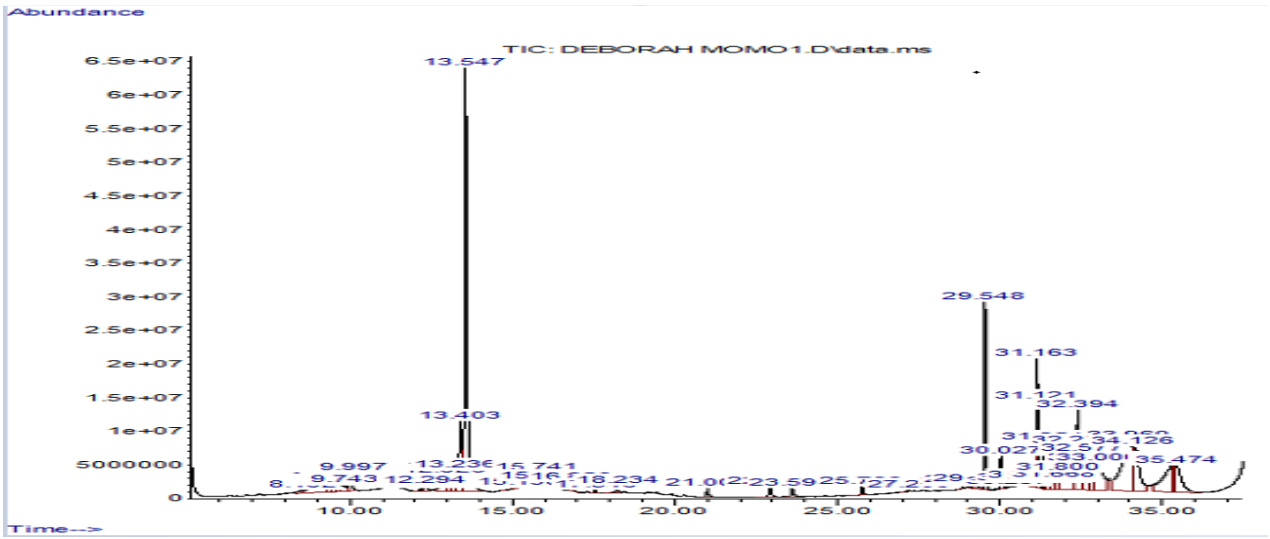

Figure 3: Chemical structures of some compounds found in OL

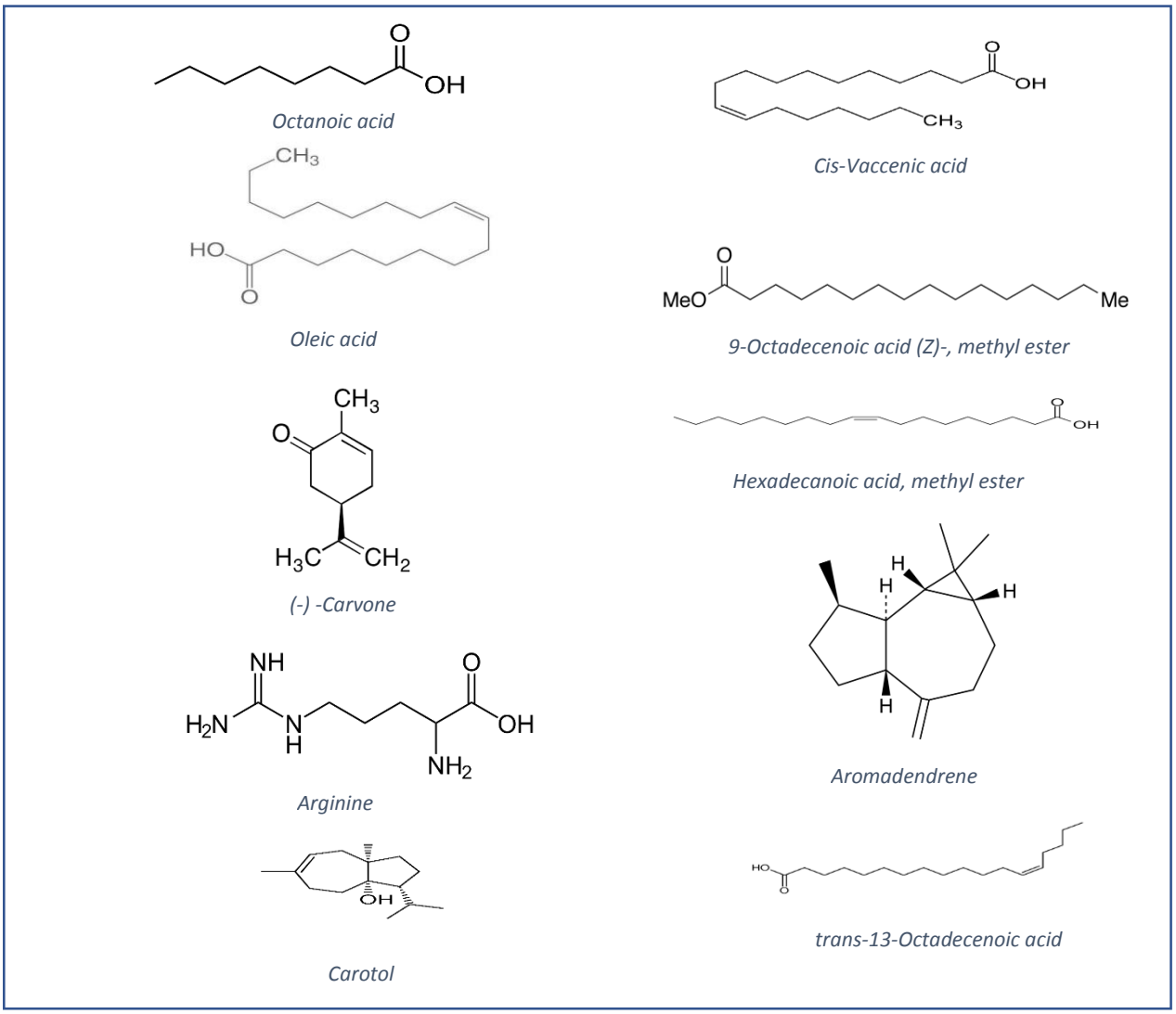

Table 2: Analgesic effect of methanol extract of OL by acetic acid induced writhing model in mice

Treatment	Dose (mg/kg)	Number of writhes	Percentage inhibition (%)
Tween 80 (control)	10.0 ml/kg	101.0 ± 4.18	-
Aspirin	100 mg/kg	79.6 ± 4.39	21.18
O. *longinoda* (200 mg)	200 mg/kg	71.4 ± 4.02	29.30
O. *longinoda* (400 mg)	400 mg/kg	47.8 ± 4.32	52.67

Table 3: Analgesic effect of methanol extract of OL by hot-plate model

Treatments	Dose	0 min	60 min	90 min	120 min	150 min	180 min
Tween 80	10 ml/kg	5.70 ± 1.32	6.40 ± 0.98	5.88 ± 0.22	4.52 ± 1.12	3.40 ± 0.85	3.63 ± 0.78
Pentazocine	10 ml/kg	5.30 ± 1.58	8.38 ± 0.56	7.88 ± 1.50	8.73 ± 0.63	9.56 ± 0.65	8.52 ± 0.97
O. *longinoda*	200 ml/kg	4.72 ± 1.81	8.34 ± 0.69	8.63 ± 0.57	8.68 ± 2.27	9.72 ± 2.29	8.70 ± 2.19
O. *longinoda*	400 ml/kg	5.62 ± 1.31	9.82 ± 3.59	13.05 ± 0.45*	8.92 ± 1.33	8.94 ± 0.89	8.79 ± 1.07

Each data represents the mean ± S.D. of 5 mice. *Significantly different from the negative control by $p < 0.05$.

Formalin induced pain in mice: Formalin induced severe pain that was split into two periods as follows: Formaldehyde directly activates nerve terminals in the initial phase (0 - 5 min) and inflammatory mediators are generated and released in the second phase (15 - 40 min). The results from the formalin induced pain method are presented in **Table 4**. The results show highly significant inhibitory effect of 200 mg/kg and 400 mg/kg of the extract in both phases (inflammatory pain), when compared to the control. When compared with pentazocine, the inhibitory effect was significantly higher at the 400 mg/kg dose in the first phase and at 200 and 400 mg/kg doses of the extracts in the second phase.

Table 4: Effect of methanolic extract of OL on formalin induced pain

Treatment (dose)	**First phase 0 - 5 min**	**Second phase 15 -30 min**
Tween 80	1.56 ± 0.01	4.08 ± 0.04
Pentazocine (10 mg)	1.28 ± 0.02	2.44 ± 0.04
O. longinoda (200 mg)	1.23 ± 0.02	2.33 ± 0.04
O. longinoda (400 mg)	1.06 ± 0.01	2.12 ± 0.02

Discussion

Oleic acid, a naturally occurring monounsaturated fatty acid interacts with the vanilloid (capsaicin)-binding pocket to suppress TRPV1(Transient receptor potential vanilloid 1) activity, pain and itch responses. This promotes the stabilization of a closed state conformation [24]. By changing the production of inflammatory mediators, modulating neutrophil infiltration and changing the VEGF effector pathway, the observed anti-inflammatory effects of oleic acid, mead acid (an omega-9 fatty acid) and erucic acid were intended to reduce inflammation in a variety of physiological and pathological conditions and be effective for wound healing and eye inflammation [25]. In a study conducted by Harima et al. [26]. Arginine was found to slightly relieve pain within 15 min after the onset of infusion and at 30 min after that. Another study suggests that L-arginine improved endothelium-dependent vasodilation of coronary microcirculation in patients with microvascular angina pectoris [27].

Furthermore, aromadendrene has been found to have analgesic, antispasmodic and anti-allergic properties. It has also been proven successful in treating a number of skin disorders, including psoriasis and eczema [28] which helps to corroborate the findings of this study. In acetic acid-induced writhing model, central and peripheral analgesics are chemically induced nociception. Prostaglandins in peritoneal exudates significantly increase and it can cause abdominal constriction in mice that is associated with prostaglandin-sensitive nociceptors [29]. In hot-plate model (nociception caused by heat) is mostly used to determine whether drugs have any effect on central pain [30]. Numerous animal species are thought to be selective for opioid-like compounds when using the hot plate method [31]. For formalin induced pain, weak analgesics can be studied using the licking reaction that formalin causes in mice. Formalin induced severe pain that was split into two periods including formaldehyde directly activates nerve terminals in the initial phase and inflammatory mediators are generated and released in the late phase [32, 33].

This study indicates that the extract has the ability to inhibit acetic acid induced writhes. All of the doses had an inhibitory impact that is more potent than of the reference analgesic (aspirin) used. The extract has some analgesic properties which are thought to involve local peritoneal cells and mediated via the prostaglandin pathways. This is accomplished by inhibiting production and release of prostaglandins [34]. Hence the extract's activity might be peripherally mediated, although this will have to be further confirmed by another model. In the hot plate test of analgesia, all methanolic extract significantly

shortened the mean reaction time compared to the control. The increase of time responses after extract administration is an evidence that the extract has centrally acting analgesic function, as indicated by the increase in reaction time. The ant's central analgesic effect was further confirmed using the formalin induced pain in mice. This shows that the aqueous extract of OL significantly increased the mean reaction time compared with the control. It can be inferred, therefore, from the results obtained that the extract possesses some centrally acting analgesic activity which was previously seen in the hot plate and acetic acid induced writhing models. It is known that centrally acting analgesic drugs evaluate the pain threshold of mice towards heat which increases the mean reaction time [35]. The activation of some receptors, such as Mu receptors in the central nervous system, which, when excited, have the intrinsic capacity to diminish the effective components of pain, may help to explain this rise in the mean reaction time. They might also be brought on by the effects of medications that have the ability to activate these receptors in higher brain areas [36]. The extract significantly sped up the mice's response times in the formalin-induced pain test neurogenic pain and inflammatory pain consistent with earlier findings. Pentazocine, the commonly used centrally acting analgesic, was found to have an impact that was just as substantial, if not more so, at a larger dose. Formalin causes two types of pain: neurogenic pain which is caused by the production of substance P and inflammatory pain which caused by release of histamine, serotonin, prostaglandins and bradykinin [37]. It appears that the aqueous extract OL reduces all phases of the pain caused by formalin indicating that it may function as narcotic analgesic and non-steroidal anti-inflammatory medication. The extract impact was more significant in the second phase than in the first phase according to the analysis of the data. This finding supports the possibility that the extract's analgesic impact is peripherally mediated through suppression of the mediators such as prostaglandins, histamine, bradykinins and other mediators [37]. As demonstrated by the hot plate and acetic acid models as well as the formalin test which has been suggested as a model for chronic pain and is responsive to centrally active analgesics, the aqueous extract of OL may also have some central analgesic effects. Thus, it follows that the ant extract of OL will be useful in treating central/neurogenic pain and inflammatory pain caused by conditions like rheumatoid arthritis. Overall, it was determined that OL is safe to consume due to its low levels of toxicity even at high dosages.

Conclusion: The study revealed the presence of bioactive chemical constituents (alkaloids, flavonoids, steroids among others). It also proved that OL has potent analgesic activity is safe to be used. The findings corroborate the traditional use of the ants as therapeutics. Further, molecular and cellular studies should be conducted to examine OL modes of action or mechanism and a detailed data should be obtained for each active ingredient.

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